UNITED STATES PATENT AND TRADEMARK OFFICE
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CATEGORY:

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Customer Number: 000959

DIVISIONAL-CONTINUATION APPLICATION TRANSMITTAL FORM UNDER RULE 1.53(b) (former Rule 1.60)

DOCKET NUMBER	ANTICIPATED CLASSIFICATION OF THIS APPLICATION:		PRIOR APPLICATION SERIAL NUMBER: 08/902,247	PRIOR APPLICATION FILING DATE: JULY 29, 1997
STI-201CN2	CLASS:	SUBCLASS:	EXAMINER: J. KERR	ART UNIT: 1633

ASSISTANT COMMISSIONER FOR PATENTS
BOX PATENT APPLICATION
WASHINGTON, DC 20231

CERTIFICATION UNDER 37 CFR 1.10

Date of Deposit: August 3, 2000 Mailing Label Number: EL 373 306 038 US

I hereby certify that this 37 CFR 1.53(b) request and the documents referred to as attached therein are being deposited with the United States Postal Service on the date indicated above in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR 1.10 and addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Larry Taylor
Name of Person Mailing Paper

Signature of Person Mailing Paper

Dear Sir:

This is a request for filing a ☒ continuation ☐ divisional application under 37 CFR 1.53(b), of pending prior application serial no. 08/902,247 filed on July 29, 1997, of Clifford A. Lingwood, *et al.* entitled Verotoxin Pharmaceutical Compositions and Medical Treatments Therewith which in turn is a continuation application of serial no. 08/386,957 filed on February 10, 1995.

1. ☒ Enclosed is a copy of the latest inventor signed application, including the oath or declaration as originally filed. The copy of the enclosed papers is as follows:
   - ☒ 29 page(s) of specification
   - ☒ 1 page(s) of claims
   - ☒ 1 page(s) of abstract
   - ☒ 6 sheet(s) of informal drawing (Figs. 1-9)
   - ☒ 1 page(s) of copy of executed declaration and power of attorney.

   I hereby verify that the attached papers are a true copy of the prior complete application serial no. 08/902,247 as originally filed on July 29, 1997.

2. ☒ Two verified statements to establish small entity status under 37 CFR 1.9 and 1.27, copies of which are enclosed, were filed in the prior application and such status is still proper and desired (37 CFR 1.28(a)).

3. ☒ The filing fee is calculated below:

	NUMBER OF CLAIMS FILED			NUMBER EXTRA
TOTAL	* 10	MINUS	** 20	= 0
INDEP.	* 1	MINUS	*** 3	= 0
☐ MULTIPLE DEPENDENT CLAIMS				

SMALL ENTITY			OTHER THAN A SMALL ENTITY	
RATE	FEE	OR	RATE	FEE
x 9 =	$0.00		x 18 =	$.00
x 39 =	$0.00		x 78 =	$.00
+130 =	$0.00		+ 260 =	$.00
BASIC FEE	$345.00		BASIC FEE	$.00
TOTAL	$345.00	OR	TOTAL	$0.00

4. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required in connection with this communication, or credit any overpayment, to Deposit Account No. 12-0080. A duplicate copy of this sheet is enclosed.

5. ☒ A check in the amount of $345.00 is enclosed for payment of the filing fee.

6. ☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

7. ☒ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claims in the prior application.)

8. ☒ Amend the specification by inserting before the first line the sentences: "This application is a continuation application of serial no. 08/902,247 filed on July 29, 1997, Issuing, which in turn is a continuation application of serial no. 08/386,957 filed on February 10, 1995. The contents of all of the aforementioned application(s) are hereby incorporated by reference."

9. ☐ Please abandon said prior application as of the filing date accorded this application. A duplicate copy of this transmittal is enclosed for filing in the prior application file. (May be used if signed by person authorized by §1.138 and before payment of base issue fee.)

10. ☒ Transfer the drawings from the pending prior application to this application.

11. ☒ Priority of application serial no. 2,116,197 filed on February 22, 1994 in Canada is claimed under 35 U.S.C. §119.
    - ☐ The certified copy has been filed in prior application serial no. _____ filed on _____.
    - ☒ The certified copy will follow.

12. ☐

13. ☐ A _____ month extension of time has been submitted in the parent application Serial No. in order to establish copendency with the present application.

14. ☐ Also enclosed is/are .

15. ☒ The power of attorney in the prior application is to Cushman , Darby & Cushman .

    a. ☒ The power appears in the original papers in the prior application.

    b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

    c. ☐ A new power has been executed and is attached.

16. ☒ Address all future communications (May only be completed by applicant, or attorney or agent of record) to Giulio A. DeConti, Jr., Esq. at **Customer Number: 000959** whose address is:

Lahive & Cockfield, LLP
28 State Street
Boston, Massachusetts 02109

17. ☒ Any requests for extensions of time necessary in a parent application for establishing copendency between this application and a parent application are hereby requested and the Commissioner is authorized to charge any fee associated with such an extension to Deposit Account No. 12-0080.

18. ☐ Pursuant to 37 CFR 1.821(e), the computer readable form of the sequence listing for this new application is to be identical with the computer readable form of application serial no._____. Please use the computer readable form of application serial no._____ in lieu of filing a duplicate computer readable form in this application. Pursuant to 37 CFR 1.821(f), the content of the paper copy of the sequence listing for this new application and the computer readable form of application serial no._____ are the same.

August 3, 2000
Date

LAHIVE & COCKFIELD, LLP
28 State Street
Boston, Massachusetts 02109
Tel. (617) 227-7400

Giulio A. DeConti, Jr., Esq.
Reg. No. 31,503
☐ inventor(s) ☐ filed under §1.34(a)
☐ assignee of complete interest
☒ attorney or agent of record

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Clifford A. Lingwood, Hannah Farkas-Hinsley, and Richard Hill

Serial No.: Not Yet Assigned

Filed: Herewith

For: Verotoxin Pharmaceutical Compositions and Medical Treatments Therewith

Attorney Docket No.: STI-201CN2

Group Art Unit: 1633

Examiner: J. Kerr

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATION UNDER 37 CFR 1.10

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Larry Taylor
Name of Person Mailing Paper

Signature of Person Mailing Paper

PRELIMINARY AMENDMENT

Dear Sir:

Prior to examination of the above-referenced application, Applicants request entry of the following amendments. Please amend the application as follows:

In the claims:

Cancel claims 1-10.

Add the following new claims:

CLIFFORD A. LINGWOOD, HANNAH FARKAS-HIMSLEY (deceased)
Inventor(s): AND ~~ROBERT~~ HILL
Appln. or Patent No.: Richard
Filed or Issued: ~~February 10, 1995~~
For: VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND MEDICAL TREATMENTS THEREWITH

(Atty. Dkt. ~~217380/ SL427~~ M# / Client Ref.

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND MEDICAL TREATMENTS THEREWITH described in

[X] the specification filed herewith.
[ ] application No. 0 /_____, filed _____.
[ ] patent No. _____, issued _____.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, convey or license any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each (small entity) person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention:

[ ] no such person, concern, or organization
[X] persons, concerns or organizations listed below in (A) and (B)

(A) FULL NAME of assignee/licensee/grantee/conveyee* UNIVERSITY OF TORONTO INNOVATIONS FOUNDATION
ADDRESS 525 UNIVERSITY AVE., TORONTO, ONTARIO, CANADA M5G 2L3
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [X] NONPROFIT ORGANIZATION

(B) FULL NAME of assignee/licensee/grantee/conveyee* _____
ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

*NOTE: Separate verified statement is required from each person, concern or organization named in (A) and (B) above having rights to the invention, averring to his/her/its status as a small entity. (37 CFR 1.27)

I acknowledge the duty to file, in this case, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

1. CLIFFORD A. LINGWOOD
NAME OF INVENTOR
Signature of Inventor
23.11.94
Date

2. HANNAH FARKAS-HIMSLEY (deceased)
NAME OF INVENTOR
Estate of H. Farkas-Himsley
By:
Signature of Inventor
24.11.94
Date

3. RICHARD ~~ROBERT~~ HILL
NAME OF INVENTOR
Richard J Hill
Signature of Inventor
24 Nov 94
Date

CDC-138 1/91(PC)

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - **NONPROFIT ORGANIZATION**

...reby declare that I am an official empowered to act on behalf of the nonprofit organization dentified below:

NAME OF ORGANIZATION UNIVERSITY OF TORONTO INNOVATIONS FOUNDATION
ADDRESS OF ORGANIZATION 525 UNIVERSITY AVENUE, SUITE 925
TORONTO, ONTARIO, CANADA M5G 2L3

:YPE OF ORGANIZATION

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

hereby declare that the nonprofit organization identified above qualifies as a nonprofit ganization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) d (b) of Title 35, United States Code, with regard to the invention
titled VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND MEDICAL TREATMENTS THEREWITH
inventor(s) CLIFFORD A. LINGWOOD, HANNAH FARKAS-HIMSLEY (deceased) & ROBERT HILL described in

→ [X]the Specification filed herewith,
e → [ ]Application No. 0 /_____, filed _____.
x → [ ]Patent No. _____, issued _____.

hereby declare that rights under contract or law have been conveyed to and remain with the nprofit organization with regard to the above identified invention.

the rights held by the nonprofit organization are not exclusive, each small entity dividual, concern or organization having rights to the invention is listed below in (A) and) and no rights to the invention are held by any person, other than the inventor, who could t qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not alify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 9(e).

) FULL NAME of assignee/licensee/grantee/conveyee* _____
ADDRESS _____
proper box: [ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

) FULL NAME of assignee/licensee/grantee/conveyee* _____
ADDRESS _____
proper box: [ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

*NOTE: Separate verified statement is required from each person, concern or organization named in (A) and (B) above having rights to the invention, averring to his/her/its status as a small entity. (37 CFR 1.27)

...knowledge the duty to file, in this case, notification of any change in status resulting in ss of entitlement to small entity status prior to paying, or at the time of paying, the ...liest of the issue fee or any maintenance fee due after the date on which status as a small ...ity is no longer appropriate. (37 CFR 1.28(b))

...hereby declare that all statements made herein of my own knowledge are true and that all ...tements made on information and belief are believed to be true; and further that these ...ments were made with the knowledge that willful false statements and the like so made are ...hable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States ...e, and that such willful false statements may jeopardize the validity of the application, any ...tent issuing thereon, or any patent to which this verified statement is directed.

ME OF PERSON SIGNING EDWARD J. KENNEY
TLE IN ORGANIZATION PRESIDENT
DRESS OF PERSON SIGNING 525 UNIVERSITY AVE., SUITE 925, TORONTO, ONTARIO, CANADA M5G 2L3
GNATURE Stewart Kenney DATE 28 NOV. 1989

...C-142 8/89

11. A method for killing cancer cells from a patient, comprising obtaining said cells from said patient, contacting said cells with a verotoxin, such that said cells are killed.

12. The method of claim 11, wherein said cells are killed *in vitro.*

13. The method of claim 11, wherein said cells are Gb_3 positive.

14. The method of claim 11, wherein said patient is a human.

15. The method of claim 11, wherein said verotoxin is verotoxin 1.

16. The method of claim 11, wherein said verotoxin is verotoxin 2.

17. The method of claim 11, wherein said verotoxin is verotoxin 2c.

18. The method of claim 11, wherein said cancer cells are from a tumor.

19. The method of claim 18, wherein said tumor is a breast, ovarian, brain, or skin tumor.

20. The method of claim 11, wherein said cells are multidrug resistant.

REMARKS

Claims 1-10 were pending and have been canceled. Claims 11-20 have been added and are currently pending. No new matter has been added. Support for new claim 11 can be found in the originally filed specification, for example, at least at page 26, line 20 through page 27, line 4. Support for new claim 12 can be found in the originally filed specification, for example, at least at page 26, lines 20-21. Support for new claim 13 can be found in the originally filed specification, for example, at least at page 27, lines 5-25. Support for new claim 14 can be found in the originally filed specification, for example, at least at page 27, lines 5-6. Support for new claims 15-17 can be found in the originally filed specification, for example, at least at page 27, lines 34-36. Support for new claims 18 - 20 can be found in the originally filed specification, for example, at least at page 26, lines 20 through page 27, line 28, line 14. No new matter has been added.

SUMMARY

The cancellation of the claims is being made solely to expedite prosecution of the above-identified application. Applicants reserve the option to further prosecute the same or similar claims in the present or another patent application. If a telephone conversation with Applicants' Attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

Date: August 3, 2000

LAHIVE & COCKFIELD, LLP
Attorneys at Law

By _____
Giulio A. DeConti, Jr.
Reg. No. 31,503
28 State Street
Boston, MA 02109
(617) 227-7400
(617) 742-4214

SL427

VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND MEDICAL TREATMENTS THEREWITH

Field of the Invention

This invention relates to verotoxin pharmaceutical compositions and to methods of treating mammalian neoplasia, particularly, ovarian and skin cancers, therewith.

Background to the Invention

Bacteriocins are bacterial proteins produced to prevent the growth of competing microorganisms in a particular biological niche. A preparation of bacteriocin from a particular strain of E. coli (HSC_{10}) has long been shown to have anti-neoplastic activity against a variety of human tumour cell lines in vitro (1,2). This preparation, previously referred to as PPB (partially purified bacteriocin (2)) or ACP (anti-cancer proteins (2)) was also effective in a murine tumour model, of preventing metastases to the lung (2).

Verotoxins, also known as SHIGA-like toxins, comprise a family known as Verotoxin 1, Verotoxin 2, Verotoxin 2c and Verotoxin 2e of subunit toxins elaborated by some strains of E. coli (3). These toxins are involved in the etiology of the hemolytic uremic syndrome (3,4) and haemorrhagic colitis (5). Cell cytotoxicity is mediated via the binding of the B subunit of the holotoxin to the receptor glycolipid, globotriaosylceramide, in sensitive cells (6).

The verotoxin family of E coli elaborated toxins bind to the globo series glycolipid globotriaosylceramide and require terminal gal α-1-4 gal residue for binding. In addition, VT2e, the pig edema disease toxin, recognizes globotetraosylceramide (Gb_4) containing an additional β 1-3 linked galNac residue. These glycolipids are the functional receptors for these toxins since incorporation of the glycolipid into receptor negative cells renders the recipient

cells sensitive to cytotoxicity. The toxins inhibit protein synthesis via the A subunit - an N-glycanase which removes a specific adenine base in the 28S RNA of the 60S RNA ribosomal subunit. However, the specific cytotoxicity and specific activity is a function of the B subunit. In an in vitro translation system, the verotoxin A subunit is the most potent inhibitor of protein synthesis yet described, being effective at a concentration of about 8 pM. In the rabbit model of verocytotoxemia, pathology and toxin targeting is restricted to tissues which contain the glycolipid receptor and these comprise endothelial cells of a subset of the blood vasculature. Verotoxins have been strongly implicated as the etiological agents for hemolytic uremic syndrome and haemorrhagic colitis, microangiopathies of the glomerular or gastrointestinal capillaries respectively. Human umbilical vein endothelial cells (HUVEC) are sensitive to verotoxin but this sensitivity is variable according to cell line. Human adult renal endothelial cells are exquisitely sensitive to verotoxin in vitro and express a correspondingly high level of Gb_3. However, HUS is primarily a disease of children under three and the elderly, following gastrointestinal VTEC infection. It has been shown that receptors for verotoxin are present in the glomeruli of infants under this age but are not expressed in the glomeruli of normal adults. HUVEC can be sensitized to the effect of verotoxin by pretreatment by tumour necrosis factor which results in a specific elevation of Gb_3 synthesis (7,8). Human renal endothelial cells on the other hand, although they express high levels of Gb_3 in culture, cannot be stimulated to increase Gb_3 synthesis (8). It has been suggested that the transition from renal tissue to primary endothelial cell culture in vitro results in the maximum stimulation of Gb_3 synthesis from a zero background (9). We therefore suspect that HUS in the elderly is the result of verotoxemia and a concomitant stimulation of renal endothelial cell Gb_3 synthesis by some other factor, eg. LPS stimulation of serum α TNF. Thus under

these conditions, the majority of individuals (excepting the very young) would not be liable to VT induced renal pathology following systemic verotoxemia.

It has also shown that the verotoxin targets a sub-population of human B cells in vitro (10). These Gb_3 containing B cells are found within the germinal centres of lymph nodes (11). It has been proposed that Gb_3 may be involved in a germinal centre homing by CD19 positive B cells (12) and that Gb_3 may be involved in the mechanisms of antigen presentation (13).

Elevated levels of Gb_3 have been associated with several other human tumours (14-16), but ovarian tumours have not been previously investigated. Gb_3 is the p^k blood group antigen (17). Tissue surveys using anti-p^k antisera have shown that human ovaries do not express this glycolipid (18, 19).

Sensitivity to VT1 cytotoxicity in vitro has been shown to be a function of cell growth, the stationary phase cells being refractile to cytotoxicity (20). The sequence homology between the receptor binding B subunit and the human α2-interferon receptor and the B cell marker CD19 suggests that expression of Gb_3 is involved in the mechanism of α2-interferon and CD19 signal transduction (12). On surface ligation, Gb_3 has been shown to undergo a retrograde intracellular transport via the rough endoplasmic reticulum to the nuclear membrane (21).

Reference List

The present specification refers to the following publications, each of which is incorporated herein by reference:

1. Farkas-Himsley, H. and R. Cheung. Bacterial Proteinaceous Products (bacteriocins as cytotoxic agents of neoplasia). Cancer Res. 36:3561-3567, (1976).

2. Hill, R.P. and H. Farkas-Himsley. Further studies of the action of a partially purified bacteriocin against a murine fibrosarcoma. Cancer Res. 51:1359-1365 (1991).

3. Karmali, M.A. Infection by Verocytotoxin-producing Escherichia coli. Clin. Microbiol. Rev. 2:15-38 (1989).

4. Karmali, M.A., M. Petric, C. Lim, P.C. Fleming, G.S. Arbus and H. Lior, 1985. The association between hemolytic uremic syndrome and infection by Verotoxin-producing Escherichia coli, J. Infect. Dis. 151:775.

5. Riley, L.W., R.S. Remis, S.D. Helgerson, H.B. McGee, J.G. Wells, B.R. Davis, R.J. Hebert, E.S. Olcott, L.M. Johnson, N.T. Hargrett, P.A. Blake and M.C. Cohen. Haemorrhagic colitis associated with a rare Escherichia coli serotype. N. Engl. J. Med. 308:681 (1983).

6. Lingwood, C.A., Advances in Lipid Research. R. Bell, Y.A. Hannun and A.M. Jr. Academic Press. 25:189-211 (1993).

7. van de Kar, N.C.A.J., L.A.H. Monnens, M. Karmali and V.W.M. van Hinsbergh. Tumour necrosis factor and interleukin-1 induce expression of the verotoxin receptor globotriaosyl ceramide on human endothelial cells. Implications for the pathogenesis of the Hemolytic Uremic Syndrome. Blood. 80:2755, (1992).

8. Obrig T., C. Louise, C. Lingwood, B. Boyd, L. Barley-Maloney and T. Daniel. Endothelial heterogeneity in Shiga toxin receptors and responses. J. Biol. Chem. 268:15484-15488 (1993).

9. Lingwood, C.A. Verotoxin-binding in human renal sections. Nephron. 66:21-28 (1994).

10. Cohen, A., V. Madrid-Marina, Z. Estrov, M. Freedman, C.A. Lingwood and H.M. Dosch. Expression of glycolipid receptors to Shiga-like toxin on human B lymphocytes: a mechanism for the failure of long-lived antibody response to dysenteric disease. Int. Immunol. 2:1-8 (1990).

11. Gregory, C.D., T. Turz, C.F. Edwards, C. Tetaud, M. Talbot, B. Caillou, A.B. Rickenson and M. Lipinski. 1987. Identification of a subset of normal B cells with a Burkitt's lymphoma (BL)-like phenotype. J. Immunol. 139:313-318 (1987).

12. Maloney, M.D. and C.A. Lingwood, CD19 has a potential CD77 (globotriaosyl ceramide) binding site with sequence similarity to verotoxin B-subunits: Implications of molecular mimicry for B cell adhesion and enterohemorrhagic E. coli pathogenesis. J. Exp. Med. 180: 191-201, (1994).

13. Maloney, M. and C. Lingwood. Interaction of verotoxins with glycosphingolipids. TIGG. 5:23-31 (1993).

14. Li, S.C., S.K. Kundu, R. Degasperi and Y.T. Li. Accumulation of globotriaosylceramide in a case of leiomyosarcoma. Biochem. J. 240:925-927 (1986).

15. Mannori G., O. Cecconi, G. Mugnai and S. Ruggieri. Role of glycolipids in the metastatic process: Characteristics neutral glycolipids in clones with different metastatic potentials isolated from a murine fibrosarcoma cell line. Int. J. Cancer. 45:984-988 (1990).

16. Ohyama, C., Y. Fukushi, M. Satoh, S. Saitoh, S. Orikasa, E. Nudelman, M. Straud and S.I. Hakomori. Changes in glycolipid expression in human testicular tumours. Int. J. Cancer. 45:1040-1044, (1990).

17. Naiki, M. and D.M. Marcus. Human erythrocyte P and P^k blood group antigens: Identification as glycosphingolipids. Biochem. Biophys. Res. Comm. 60:1105-1111, (1974).

18. Pallesen, G. and J. Zeuthen. Distribution of the Burkitt's-lymphoma-associated antigen (BLA) in normal human tissue and malignant lymphoma as defined by immunohistological staining with monoclonal antibody 38:13. J. Cancer Res. Clin. Oncol. 113:78-86 (1987).

19. Kasai, K., J. Galton, P. Terasaki, A. Wakisaka, M. Kawahara, T. Root and S.I. Hakomori. Tissue distribution of the Pk antigen as determined by a monoclonal antibody. J. Immunogenet. 12:213 (1985).

20. Pudymaitis, A. and C.A. Lingwood. Susceptibility to verotoxin as a function of the cell cycle. J. Cell Physiol. 150:632-639 (1992).

21. Sandvig, K., O. Garred, K. Prydz, J. Kozlov, S. Hansen and B. van Deurs. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature. 358:510-512 (1992).

Although anti-neoplastic effects of bacterial preparations have been known for over twenty years, the neoplastic effect of verotoxin per se has, to-date, remained unknown. As a result of extensive investigations, we have discovered that verotoxin, particularly Verotoxin 1, is an active component within the ACP and that purified Verotoxin 1 has potent anti-neoplasia effect in vitro and in vivo. Most surprisingly, we have found effective in vivo anti-cancer treatments of human beings commensurate with non-toxic administered dosages.

Summary of the Invention

It is an object of the present invention to provide a pharmaceutical composition for the treatment of mammalian neoplasia and, particularly, skin cancer and ovarian cancer.

It is a further object of the present invention to provide a method of treating mammalian neoplasia, particularly, skin, brain and ovarian cancers.

Accordingly, in one aspect the invention provides a pharmaceutical composition for the treatment of mammalian neoplasia comprising a non-lethal anti-neoplasia effective amount of a verotoxin, preferably, verotoxin 1, and a suitable pharmaceutically acceptable diluent, adjuvant or carrier therefor.

The invention preferably provides a pharmaceutical composition and method of treatment for mammalian skin cancers, brain cancers and ovarian cancer.

In a further aspect the invention provides a process for the manufacture of a pharmaceutical composition for the treatment of mammalian neoplasia, said process comprising admixing verotoxin with a pharmaceutically acceptable carrier, adjuvant or diluent therefor.

The present invention provides selective, specific cancer treatments wherein verotoxin selectively binds with Gb_3 in Gb_3-containing cells. This is in contrast to the use of broad spectrum anti-neoplastic agents such as most chemotherapeutic agents, in that non-Gb_3 containing cells are not affected by verotoxin. The present invention thus provides a most beneficial, cell-selective, therapeutic treatment.

The treatment is of value against cutaneous T-cell lymphomas, particularly, Mycosis Fungoides, sezary syndrome and related cutaneous disease lymphomatoid papilosis. For example, Mycosis fungoides lesions in humans have been cleared without any observed adverse systemic effects by the application of VT1 (5ng in 2 ml. solution) by interdermal injection in patients.

In a further aspect, the invention provides a method of treating mammalian neoplasia comprising treating said mammal with a non-lethal anti-neoplasia effective amount of a verotoxin, preferably Verotoxin 1.

The verotoxin may be administered to the patient by methods well-known in the art, namely, intravenously, intra-arterially, topically, subcutaneously, by ingestion, intra-muscular injection, inhalation, and the like, as is appropriately suitable to the disease. For treatment of a skin cancer, sub-cutaneous application is preferred.

In the practice of the present invention, Verotoxin 1 has been injected intramuscularly into a patient with advanced ovarian carcinoma. No adverse affects were

monitored on lymphocyte or renal function and a serum tumour marker was found to continue to rise when the patient was treated with relatively high doses of Verotoxin 1. This tumour was refractory to all conventional cancer therapies. No effect was found on hemoglobin levels.

The verotoxin is, typically, administered in a suitable vehicle in which the active verotoxin ingredient is either dissolved or suspended in a liquid, such as serum to permit the verotoxin to be delivered for example, in one aspect from the bloodstream or in an alternative aspect subcutaneously to the neoplastic cells. Alternative, for example, solutions are, typically, alcohol solutions, dimethyl sulfoxide solutions, or aqueous solutions containing, for example, polyethylene glycol containing, for example, polyethylene glycol 400, Cremophor-EL or Cyclodextrin. Such vehicles are well-known in the art, and useful for the purpose of delivering a pharmaceutical to the site of action.

Several multi-drug resistant cell lines were found to be hypersensitive to Verotoxin 1. For example, multidrug resistant ovarian cancer cell lines SKVLB and SKOVLC were more sensitive to VT cytotoxicity than corresponding non-multidrug resistant ovarian cancer cell line SKOV3. Such an observation indicates the possible beneficial effect for patients bearing the SKVLB cell line cancer than those with the SKOV3 cell line under VT treatment. Further, our observed binding of VT1 to the lumen of blood vessels which vascularize the tumour mass, in addition to the tumour cells per se, may result in an anti-angiogenic effect to augment the direct anti-neoplastic effect of verotoxin.

Brief Description of the Drawings

In order that the invention may be better understood preferred embodiments will now be described, by

way of example only, with reference to the accompanying drawings wherein:

Fig. 1 shows the selective neutralization of ACP cytotoxicity by anti VT1 and or anti VT1 B subunit but not by anti VT2 antibodies as determined by cell density measurement after 48 hours;

Fig. 2 shows the viability of selected ovarian and breast tumour cell lines to verotoxin concentration;

Fig. 3 represents VT1 contained within ACP preparation binding to Gb_3 (and Gb_2).

Fig. 4 represents VT thin layer chromatography overlay of ovarian tumour and ovary glycolipids;

Fig. 5 represents VT thin layer chromatography overlay of selected cell line glycolipids;

Fig. 6 represents in three graphs ovarian cell line sensitivity to VT1, VT2 and VT2c;

Fig. 7 represents glioblastoma multiforme cell line sensitivity to VT1, VT2 and VT2c;

Fig. 8 represents the distribution of labelled VT1 B subunit (VTB-^{131}I) administered IP (inter-peridinually) in a Gb_3 tumour bearing nude mouse;

and Fig. 9 represents the results of a three-day treatment of several human astrocytoma cell lines with VT1.

Detailed Description of the Invention

Experimental

The isolation and purification of verotoxins VT1, VT2 and VT2c have been earlier described.

Verotoxin 1 was prepared genetically from the high expression recombinant E. coli pJB28, J. Bacteriol 166:375 and 169:4313. The generally protein purification procedure described in FEMS Microbiol. Lett. 41:63, was followed.

Verotoxin 2 was obtained from R82, Infect. Immun. 56:1926-1933; (1988); and purified according to FEMS Microbiol. Lett. 48:379-383 (1987).

Verotoxin 2c was obtained from a clinical strain E32511 and purified according to FEMS Microbiol. Lett. 51:211-216 (1988).

Purification of VT1 from JB28

Pellet Preparation may be conducted as follows:

1. Prepare 6 x 1L LB broth in 3 x 5L jugs (media) and autoclave.
   Add carbenicillin to give a 100 μg/ml final conc. when cool.
2. Seed at least 6 ml of penassay (tubes in cold room) + 100 μg/ml carbenicillin with JB28 and incubate O/N @ 37°C.
3. Seed jugs (1 ml seed/litre broth) next morning and incubate for 24 hours at 37°C at 200 rpm (vigorous shaking).
4. Spin down bugs at 9K for 15 min. at 4°C and scrape pellet into a freezer bag for future use. Freeze at -70°C.

Preparation of Crude Toxin Extract:

1. Retrieve pellet and dump into beaker. Resuspend in 400 ml of PBS containing 0.1 mg/ml polymyxin B, 50 mg PMSF using a blender. Blend thoroughly then sonicate on ice for - 1 minute to disperse further.
2. Incubate in shaking incubator, 200 rpm, or with vigorous stirring @37°C for 1 hour.
3. Spin down cells @ 9K for 15 minutes.
4. Pour off supernatant and keep. Resuspend pellet in 400 ml PBS with 0.1 mg/ml polymyxin B and PMSF. Blend and sonicate as before.
5. Incubate with vigorous shaking/stirring at 37°C for 1 hour.
6. Spin at 10K for 15 minutes and save supernatant.

7. The supernatants should be quite yellow and the bacterial pellet should become more fine and diffuse with each extraction step.
8. Filter the combined supernatants through Whatman filter paper than through a glass fibre filter to clarify. This step is optional, but will greatly speed the concentration step.
9. Amicon the combined supernatants at 70 psi (max.) using a YM10 membrane (takes about 200 hours) to concentrate to < 50 ml.

Chromatography:

Hydroxylapatite

1. Equilibrate hydroxylapatite column with 10mM K or Na phosphate (several column volumes).
2. Load sample and wash with equilibration buffer until absorbance of effluent is negligible.
3. Add 2 column volumes (150 ml) of 100mM K phosphate (until yellow-coloured fractions emerge) and collect 3 ml fractions.
4. Wash column with 500mM K phosphate and re-equilibrate with 10mM K phosphate. Add 0.05% sodium azide.

Chromatofocussing

5. Measure fractions (A_{280}) and Pool peak fractions from HA.
6. Dialyse against 2L 0.025M imidazole-HCI pH 7.4 O/N. Also equilibrate the chromatofocussing column O/N with the same (300 ml).
7. Load sample and follow with 400 ml polybuffer-HCI pH 5.0 (50ml polybuffer 74 + 350ml dH_2O, a 1:7 dilution, - pH to 5.0 with HCI). NOTE: make sure the sample is equilibrated to the temperature that the column will be run at (usually room temperature) prior to loading. If the column is to be run at 4° then buffers must be pH'd at 4°C and the column equilibrated at this temperature.

8. Collect 1 ml fractions and test them for A_{280} and pH.
9. Plot the A_{280} and pool peak fractions at about pH 6.8 for VT-1 (pool side peaks separately).
10. Clean column with 100 ml 1M NaCl. if really dirty follow with 100 ml 1M HCI, but quickly equilibrate column with imidazole. Store column in 20% ethanol in 25mM imidazole.

Cibachron blue

11. Equilibrate cibachron blue with 10mM Na phosphate buffer, pH 7.2 (100ml).
12. Load sample directly from CF and follow with 60ml of same buffer.
13. Elute with 0.5M NaCl in above buffer and collect fractions.
14. Test fractions for A_{280} and cytotoxicity and pool appropriate ones.
15. Clean column with 25ml each of 8M Urea in wash buffer and 1M NaCI in wash buffer.
16. Reequilibrate column with 10mM phosphate containing 0.1% azide.
17. Dialyse peak fractions against wash buffer with one change.
18. Lyophilize and resuspend in 1ml dH_2O.
19. Do protein assay and run SDS-PAGE to check purity.

Solutions:

HA column

potassium phosphate buffer (0.5M stock)

17.42g K_2HPO_4 up to 300 ml with dH20

6.8g KH_2PO_4 pH 7.2 with KOH

CF column

imidazole buffer

0.851g/500 ml H_2O

pH 7.4 with HCI

CB column

sodium phosphate buffer (Wash buffer-WB)
0.71g/500ml Na_2HPO_4
pH 7.2 with HAc
degas

Elution buffer
2.922g NaCI/100 ml WB

Cleaning Buffers
12.012g Urea/25 ml WB
1.461g NaCI/25ml WB

Purification of VT2 from R82

Pellet Preparation:

1. Prepare 3 x 2L penassay broth (Antibiotic Meida 3, DIFCO; pH 7.0) in 3 x 5L jugs and autoclave at 121°C for 20 minutes. Allow broth to cool to room temperature before use.
2. Seed minimum 3 x 2ml of penassay broth containing 75 µg/ml carbenicillin (Disodium salt, SIGMA) with R82 and incubate overnight at 37°C, with shaking.
3. Add 50 µg/ml carbenicillin to each of the 5L jugs (from step 1). Seed each jug with 2 ml of seed (step 2) and incubate for 24 hours at 37°C with shaking of approximately 120 rpm.
4. Heat incubator to 45°C and incubate for 30 minutes.
5. Reduce temperature to 37°C and incubate for another 3 hrs.
6. Spin down culture solution at 9,000xg for 15-20 min at 4°C. Discard supernatant and store pellets at -20°C.

Preparation of Crude Toxin Extract:

1. Resuspend pellets in 100 ml of PBS (phosphate buffered saline, OXOID; pH 7.3).
2. Add 0.3 mg/ml PMSF (phenylmethyl-sulfonyl fluoride, SIGMA) dissolved in 0.5 ml acetone to pellet solution. Let acetone evaporate. Sonicate on ice at highest

output possible for 5 min or until an homogeneous solution is obtained.

3. Spin down cell at 9,000xg at 4°C for 20 min. Discard pellets.
4. Concentrate supernatants using ultrafiltration (Model 8400 standard infiltration cell, AMICON) with N_2 no higher than 70 psi and using a 10,000 MW cutoff membrane filter (YM10 membrane, AMICON).
5. Using 12-14,000 MW cutoff tubing (SPECTRAPOR) (now and in all dialysis steps), dialyse toxin solution against 4L of 10mM potassium phosphate overnight, with stirring at 4°C.

Chromatography:

Hydroxylapatite (HA)

1. Equilibrate hydroxylapatite column (BSA binding capacity: 32 mg/g, approximately 113 ml volume; CALBIOCHEM (BEHRING DIAGNOSTICS)) with 2 column volumes of 10mM potassium phosphate.
2. Load sample and follow with 1 column volume 10mM potassium phosphate.
3. Add 2 column volumes of 200mM potassium phosphate and collect 2 ml fractions. The fractions containing the toxin should be coloured differently from the other fractions.
4. Wash column with 1 column volume of 500mM potassium phosphate and reequilibrate with 1 column volume of 10mM potassium phosphate. Add azide to the top of the column for storage.

Chromatofocussing (CF)

5. Pool peak fractions from HA column either by colour or by cytotoxicity test on Vero cells (10-fold dilutions).
6. Dialyse pooled fractions against 4L 0.025M Histidine-HCl pH 6.2 (SIGMA) overnight. Also equilibrate the

chromatofocussing column (PBE (polybuffer exchanger) 94, 1.5 cm diameter, 57 ml volume; PHARMACIA) overnight with the same buffer (300 ml).

7. Loan sample and follow with 400 ml polybuffer-HCl pH 4.0 (50 ml polybuffer 74 (PHARMACIA) + 350 ml dH_2O - pH to 4.0 with HCl).
8. Collect 2 ml fractions and test the pH of each fraction. Once the pH has dropped to 3.95, stop collecting fractions. Test the fractions using absorbance of 280 nm or by cytotoxicity on Vero cells (10-fold dilutions).
9. Pool peak fractions, and return pH to 7.0 using 1N NaOH.
10. Clean column with 200 ml 1M NaCl. If dirty follow with 100ml 1M HCl, but quickly equilibrate column with 0.025M imidazole, otherwise equilibrate with 24% EtOH-H_2O.

Cibachron blue (CB)

11. Equilibrate cibachron blue (2 cm diameter, 82 ml volume, PIERCE) with 100 ml of 10mM sodium phosphate buffer (wash buffer).
12. Load sample and follow with 60 ml of wash buffer.
13. Elute with 0.5M NaCl in wash buffer and collect 2 ml fractions.
14. Test fractions for absorbance at 280 nm using the elution buffer as a blank and cytotoxicity on Vero cells and pool appropriate fractions.
15. Clean column with 25 ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.
16. Reequilibrate column with 100 ml of wash buffer and add azide to the top of the column for storage.
17. Dialyse peak fractions against 4L 0.01M Tris-CL (pH 7.0, SIGMA).
18. Lyophilize sample and resuspend in 1-2 ml dH_2O (OPTIONAL).

19. Do protein assay (BCA Protein assay reagent, PIERCE) and rune SDS-PAGE gel (Schagger, H. and von Jagow, G.; Analytical Biochem 166, 368-379 (1987): 10% T table 2; first line table 3) to check purity.

Solutions:

HA Column

potassium phosphate buffer (0.5M stock)

17.42g K_2HPO_4 up to 300 ml with dH_2O

6.8g KH_2PO_4 pH 7.2 with KOH

CF column

Histidine buffer (0.025M)

2.0g/500 ml H_2O

pH 6.2 with HCl

CB column

Sodium phosphate buffer (Wash buffer-WB)

0.71g/500ml Na_2HPO_4

pH 7.2 with HAc

degas

Elution buffer (0.5M)

2,922g NaCl/100ml WB

Cleaning Buffers

12.01g Urea/25 ml WB

1.46 NaCl/25 ml WB

0.01 M Tris

4.84 g Trizma Base

4 L ddH_2O

pH to 7.2 with HCl

Purification of VT2c from E32511

Pellet Preparation:

1. Prepare 3 x 2L penassay broth (Antibiotic Media 3, DIFCO; pH 7.0) in 3 x 5L jugs and autoclave at 121°C for 20 minutes. Allow broth to cool to room temperature before use.

2. Seed minimum 3 x 2 ml of penassay broth with E32511 and incubate overnight at 37°C.
3. Add 0.2 μg/ml Mitomycin C (1 ml of 0.4 mg/ml) (add 5 ml of ddH_2O to the vial) to each of the 5L jugs (from step 1). Seed each jug with 2 ml of seed (step 2) and incubate for 6 hrs at 37°C with shaking of approximately 120 rpm. It is very important to stagger the incubation by about 45 min/flask because the toxin begins to deteriorate after 6 hour exposure to Mitomycin C.
4. Spin down culture solution at 9,000xg for 15-20 min at 4°C. Discard supernatant and store pellets at -20°C.

Preparation of Crude Toxin Extract:

1. Resuspend pellets in 150 ml of PBS (Phosphate buffered saline, OXOID; pH 7.3).
2. Add 0.3 mg/ml PMSF (phenylmethyl-sulfonyl fluoride, SIGMA) dissolved in 0.5 ml acetone to pellet solution. Let acetone evaporate. Sonicate on ice at highest output possible for 3 min or until an homogeneous solution is obtained.
3. Add 0.1 mg/ml polymyxin B sulphate (Aerosporin, BURROUGHS WELLCOME INC.; 500,000 units) to solution and incubate with gentle shaking at 37°C for 1 hr.
4. Spin down cells at 9,000xg at 4°C for 20 min (to remove all cells and cell debris from solution).
5. Decant supernatant and store at 4°C. Resuspend pellet in 75 ml PBS and add 0.1 mg/ml polymyxin B.
6. Incubate with gentle shaking at 37°C for 1 hr.
7. Spin down cell at 9,000xg at 4°C for 20 min and pool supernatants (from step 5). Discard pellets.

The next few steps should preferably be done at 4°C:

8. Add crystalline ammonium sulphate very slowly, with stirring to pooled supernatants to 30% saturation.

9. Let stir for 20 min and then remove precipitate by centrifugation (10000g for 10 min).
10. Add crystalline ammonium sulphate very slowly, with stirring to pooled supernatants to 70% saturation.
11. Let stir for 20 min and then centrifuge at 10000g for 10 min.
12. Resuspend pellet from step 11 in 15 ml of 0.01M Potassium phosphate buffer.
13. Using 12-14,000 MW cutoff tubing (SPECTRAPOR) (now and in all dialysis steps), dialyse toxin solution against 4L of 10mM potassium phosphate overnight, with stirring at 4°C.

Chromatography:-

Hydroxylapatite (HA)

1. Equilibrate hydroxylapatite column (BSA binding capacity: 32 mg/g, approximately 113 ml volume; CALBIOCHEM (BEHRING DIAGNOSTICS)) with 2 column volumes of 10mM potassium phosphate.
2. Load sample and follow with 1 column volume 10mM potassium phosphate.
3. Add 2 column volumes of 100mM-200mM potassium phosphate and collect 2 ml fractions. The fractions containing the toxin should be coloured differently from the other fractions.
4. Wash column with 1 column volume of 500mM potassium phosphate and reequilibrate with 1 column volume of 10mM K phosphate. Add azide to the top of the column for storage.

Chromatofocussing (CF)

5. Pool peak fractions from HA column either by colour or by cytotoxicity test on Vero cells (10-fold dilutions).
6. Dialyse pooled fractions against 4L 0.025M imidazole-HCl pH 7.4 (SIGMA) overnight. Also equilibrate the

chromatofocussing column (PBE (polybuffer exchanger) 94, 1.5 cm diameter, 57 ml volume; PHARMACIA) overnight with the same buffer (300 ml).

7. Load sample and follow with 200 ml polybuffer-HCl pH 5.0 (25 ml polybuffer 74 (PHARMACIA) + 175 ml dH_2O - pH to 5.0 with HCl).
8. Collect 2 ml fractions and test the pH of each fraction. Once the pH has dropped to 5.95, stop collecting fractions. Test the fractions for cytotoxicity on Vero cells (10-fold dilutions).
9. Pool peak fractions.
10. Clean column with 200 ml 1M NaCl. If really dirty follow with 100 ml 1M HCl, but quickly equilibrate column with 0.025M imidazole.

Cibachron blue (CB)

11. Equilibrate cibachron blue (2 cm diameter, 82 ml volume, PIERCE) with 100 ml of 10mM sodium phosphate buffer (wash buffer).
12. Load sample and follow with 60 ml of wash buffer.
13. Elute with 0.5M NaCl in wash buffer and collect 2 ml fractions.
14. Test fractions for absorbance at 280 nm using the elution buffer as a blank and cytotoxicity on Vero cells and pool appropriate fractions.
15. Clean column with 25 ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.
16. Reequilibrate column with 100 ml of wash buffer and add azide to the top of the column for storage.
17. Dialyse peak fractions against 4L 0.01M Tris-CL (pH 7.0, SIGMA).
18. Lyophilize sample and resuspend in 1-2 ml dH_2O (OPTIONAL).
19. Do protein assay (BCA Protein assay reagent, PIERCE) and run SDS-PAGE gel (Schagger, H. and von Jagow, G.;

Analytical Biochem 166, 368-379 (1987): 10% T table 2; first line table 3) to check purity.

Solutions:

HA column

potassium phosphate buffer (0.5M stock)

17.42g K_2HPO_4 up to 300 ml with dH_2O

6.8g KH_2PO_4 pH 7.2 with KOH

CF column

imidazole buffer (0.025M)

0.851g/500 ml H_2O

pH 7.4 with HCl

CB column

sodium phosphate buffer (Wash buffer-WB)

0.71g/500 ml Na_2HPO_4

pH 7.2 with HAc

degas

Elution buffer

2.922 g NaCl/100ml WB

0.01 M Tris

4.84 g Trizma Base

4 L ddH_2O

pH to 7.2 with HCl

Cleaning buffers

12.012g Urea/25ml WB

1.461g NaCl/25ml WB

Affinity purification verotoxins

500μg globotriaosyl ceramide in 1 ml chloroform was mixed and dried with 1g of dried celite. The chloroform was evaporated and the celite suspended in PBS and poured in a column. Crude polymyxin extract 20 ml (25 mg protein) the toxin producing E. coli was applied to the column and incubated at room temp for 15 mins. The column was washed with PBS and purified verotoxin eluted with 10 ml 1M Tris pH 9.6. The eluate was neutralized and dialysed. This method is applicable for purification of all verotoxins. (Boulanger, J., Huesca, M., Arab, S and Lingwood, C.A.

"Universal method for the facile production of glycolipid/lipid matrices for the affinity purification of binding ligands" Anal Biochem 217: 1-6 [1994])

Preparation of verotoxin 1 doses

VT1 was purified from the E. coli strain as previously described which overexpresses the cloned toxin genes. The purified toxin was free of endotoxin contamination. The protein concentration of this batch of verotoxin was determined and the toxin aliquoted and stored at -70°C.

To prepare VT1 doses for patients, VT1 was diluted into injection grade sterile saline containing 0.2% v/v of the patient's own serum. 210 ul of sterile patient serum was added to 10 ml of sterile injection saline and 93.9 ml of purified VT1 (6.7 g/ml) added to give a final toxin concentration of 62.5 ng/ml or 12.5 ng per 0.2 ml. dose. The final toxin preparation was sterile-filtered using a 0.2 mm syringe filter and dispensed in 2 ml aliquots into 10 ml vials. One working vial may be stored at 4°C and the remaining vials frozen until needed.

FITC labelling of VT1: FITC was added directly to VT1 (in a 1:1, w/w ratio) in 0.5M $Na_2CO_3/NaHCO_3$ conjugated buffer pH 9.5 and the mixture gently rotated for 1.2 hours at room temperature. Free FITC was removed by centricon.

Fluorescent Staining of Sections: Samples of surgically removed ovarian tumours were embedded in OCT compound, flash frozen in liquid nitrogen, and stored at -70°C until use. Five μm sections of frozen sample were thawed, allowed to dry and stained with FITC-labelled VT1 in PBS (0.5 mg.ml) containing 0.1% BSA for 1 h at room temperature. Sections were extensively washed with PBS and mounted with mounting medium containing DABCO. Sections were observed under a Polyvar fluorescent microscope.

Fluorescent Staining of Cells: Cells growing on coverslips were washed once with PBS, fixed for 2 min at room temperature with 2% formalin rinsed with PBS twice and incubated with FITC-VT1 for 1h at room temperature. The cells were washed 5 times with PBS, mounted with DABCO and observed under a Polyvar fluorescent microscope.

Quantification of VT1 antitumour activity: SKOV3 (drug sensitive human ovarian cell line), SKOVLC (SKOV3, resistant to Vincristine, and SKOVLB (SKOV3, resistant to Vinblastine) were each grown in α - MEM supplemented with 10% fetal calf-serum and tested for their sensitivity to VTs. Equal numbers of cells (approximately 1000 per/ml of media) were added to the wells of Linbro 98 well plate. 10-fold dilution of VTs were tested in triplicate and incubated for 48h at 37°C in a humidified atmosphere containing 5% CO_2. Cells were then fixed with 2% Formalin, stained with Crystal Violet, and read with ELISA plate reader.

To quantify the anticancer activity of VT1, SKOV3, SKOVLC, and SKOVLB (human ovarian cell line) were incubated with 10-fold dilution of VT1 for 48h. SKOVLC & SKOVLB (drug resistant cell lines) are more sensitive to VT1 antitumour activity than SKOV3.

Preparation of ^{131}I-VT1B

This material may be made by the following procedure.

1. Dissolve 20 mg of iodogen in 2.0 ml of chloroform (10 mg/ml). Make a 1:10 dilution by adding 0.25 ml of the 10 mg/ml solution to 2.25 ml chloroform (1 mg/ml).
2. Dispense 20 ul of this dilute solution into a clean, dry sterilized glass tub. Add 500 ul of chloroform and evaporate to dryness under N_2.
3. Add 1.5 mg. in 0.66 ml of VT1B subunit to the test tube.

4. Add 5 MCi of ^{131}I sodium iodide in 100 ul. Allow labelling to proceed for 10 mins.
5. Wash a PD-10 column with 25 ml of Sodium Chloride Injection USP.
6. Dilute ^{131}I-VT1B to 2.5 ml total volume with 1% HSA in Sodium Chloride Injection USP. Load onto PD-10 column. Elute column with 3.5 ml 1% HSA in saline.
7. Measure ^{131}I activity of eluant and column to determine LE. Draw up pooled fractions into a syringe with spinal needle attached. Detach spinal needle and attach Millex GV filter.
8. Filter into a sterile 10 ml multidose vial. Note volume filtered and assay vial for ^{131}I in dose calibrator. Calculate concentration.
9. Draw up 0.1 ml of ^{131}I-VT1B and dispense 0.05 ml into each of two 5 ml sterile multidose vials (one for sterility test and one for pyrogen test). Vials already contain 2 ml saline (=1:50 dilution).
10. Determine RCP by PC (Whatman No. 1) in 85% MeOH and by size exclusion HPLC.
11. Conduct sterility and pyrogen tests.

Fig. 1 relates to the neutralization of ACP cytotoxicity by anti-VT. KHT cell monolayers were incubated with 35 ng/ml ACP from E.coli HSC_{10}, or 10pg/ml VT1, VT2 or VT2c in the presence of monoclonal anti-VT1(PH1), monoclonal anti VT2 or polyclonal rabbit antiVT1 B subunit. The cells were incubated for 72 hours at 37°C and viable adherent cells were detected by fixation and staining with crystal violet. Cytotoxity of VT1 and ACP was completely neutralized in the presence of anti VT1 or anti VT1B subunit (anti-VT2 serum had no effect).

From measurement of the cytotoxic assay of ACP on vero cells (cells from Africa green monkey kidney that are very sensitive to verotoxin), relative to a pure VT1 standard, it was estimated that the ACP preparation contained 0.05% VT1. This concentration of purified VT1 was as

effective as ACP in inhibiting the growth of various tumour cell lines in vitro (Fig. 2). Thus, VT1 mimics the anti-neoplastic effect of ACP in vitro. VT1 was tested for the ability to inhibit the metastases of KHT fibrosarcoma cells in the mouse model as had been previously reported for ACP. The equivalent dose of VT1 was as effective as ACP, reducing the number of lung metastases to background levels, following a primary subcutaneous tumour inoculum (Table 1).

Table 1. Response of KHT cells, growing as lung modules, to treatment with VT-1 or ACP.

GP	TREATMENT	# OF MICE	# OF LUNG NODULES/MOUSE	MEAN	WT LOSS /GAIN*
EXPT 1					
1	Control	9	34,24,39,47,28 ,32,26,29,34	32.6	+5%
2	ACP-0.25 ug/mouse	4	12,31,25,15	20.8	0
3	ACP-1.0 ug/mouse	6	1,2,2,5,1	2.2	0 ** (1 death)
4	ACP-4 ug/mouse	5	0,0,0,0,0	0	-13%
5	VT-1 0.009 ug/mouse	5	29,41,34,29,21	30.8	+5%
6	VT-1 0.036 ug/mouse	5	7,16,29,16,6	14.8	+5%
7	VT-1 0.144 ug/mouse	5	1,4,2,3,1	2.2	+5%
EXPT 2					
1	Control	4	15,12,8,12	11.75	<5%
2	ACP-2 ug/mouse	5	0,1,0,0,0	0.2	<5%
3	VT-1 0.1 ug/mouse	4	0,0	0	<5%*** (2 deaths)
4	VT-1B-0.2 ug/mouse	5	13,14,9,7,19	12.4	<5%
5	VT-1B-10 ug/mouse	5	8,3,9,11	6.8	<5%

Mice were treated with VT-1 or ACP(1-p) I day after cell injection (1000 KHT cells/mouse i-v).

Lung nodules counted @ 20 days after cell injection.

* Mean change in gp wi-max during 10 days (Expl 1) or 4 days (Expt 2) after VT-1 or ACP injection. Max wt loss @ days 7-8.

** Death occurred @ days 2-3 after ACP injection
*** Deaths occurred @ days 7-8

Purified VT1 was found to mimic the anti-metastatic effect of ACP on the growth of this tumour from a primary subcutaneous site. Lung metastasis was completely inhibited. Moreover, prior immunization of mice with the purified B-subunit of verotoxin completely prevented any protective effect of ACP when the animals were subsequently treated with the tumour and ACP (Table 2).

Table 2. Response of KHT lung nodules, growing to immunized mice, to treatment with VT1 or ACP.

GP	IMMUNI-ZATION*	TREATMENT	# OF MICE	# OF LUNG NODULES/ MOUSE	MEAN	WT LOSS/ GAIN*
1	None	None	6	34,47,53, 62,43,52	48.5	<5%
2	None	VT-1 -0,2 ug/mouse	5			5 deaths (dy 6-8)**
3	None	ACP-2.0 ug/mouse	5	0,1,2,0,0	0.6	-8%
4	VT-1B+FA	None	5	43,40,47, 43,23	39.2	-6%
5	VT-1B+FA	VT-1 -0,2 ug/mouse	6	26,44,49, 21,43,37	36.7	<5%
6	VT-1B+FA	ACP-2.0 ug/mouse	6	50,38,33, 41,48,50	43.3	<5%
7	FA only	None	5	44,60,19, 25,40	37.6	<5%
8	FA only	VT-1 -0,2 ug/mouse	5			5 deaths (dy 6-8)***
9	FA only	ACP -2.0 ug/mouse	5	1,1,2,1,0	1	-6%

Mice were treated with VT-1 or ACP(i-p) 1 day after cell injection (1000 KHT cells/mouse).

Lung nodules counted @ 20 days after cell injection (i-v).

*Immunization was 2 injections of VT-1B (10ug/mouse +/- Freund's Adjuvant (FA) given (i-p) 4 weeks and 2 weeks before cell injection.

** Mean change in gp wt - max during 13 days. Maximum weight loss @ day 7-8.

ACP was tested for glycolipid binding by TLC overlay using monoclonal anti-VT1 or anti-VT2c. Anti-VT1 shows extensive binding of a component within the ACP preparation to globotriaosylceramide and galabiosyl ceramide (Fig. 3). This binding specificity is identical to that reported for purified VT1(8). No binding component reactive with anti-VT2 was detected. In Fig. 3 anti VT antibodies were used to detect binding to the immobolized glycolipids. Arrows indicate position of standard (from the top) galabiosyl ceramide, globotriaosyl ceramide and globotetraosyl ceramide. Panel 1-detection using anti VT1, panel 2-detection using anti VT2c.

VT1 demonstrated <u>in vitro</u> activity against a variety of ovarian carcinoma cell lines. A large number of primary human ovarian tumour biopsies were screened for the expression of Gb_3 via TLC overlay using purified VT1. It was found that Gb_3 was barely detectable in normal ovary tissue, whereas in all cases a significant increase in expression of Gb_3 was observed in the ovarian carcinoma. Similarly, elevated levels of Gb_3 were found in acites tumour and in tumours that had metastized to the omentum, (Fig. 4) which defines lane 1, ovarian omentum metastasis; lane 2: tumour biopsy; lane 3, tumour biopsy; lanes 3-6, normal ovary; lane 7, human kidney Gb_3 standard. Surprisingly, we have found that multi-drug resistant variants of ovarian tumour cell lines were considerably more sensitive to VT1 cytotoxicity than the drug sensitive parental cell line (Figs 2, 5 and 6). Similar effects had been observed for ACP. Fig. 2 shows human ovarian tumour cell lines sensitive to ACP tested for VT sensitivity. Human ovarian and breast tumour derived cell lines were tested for VT1 sensitivity wherein ovarian 1, 2,

3, 4 and 5 are denoted ¤, +, x, ▪ and o respectively, and breast-SKBR3△, 468♦, 453●, 231▲. The cell lines 1-ovarian, 453 and SKBR3, previously shown to be resistant to ACP, were also resistant to up to 20 ng/ml VT1.

The 1, 2, 3 and 4 cells were from ovarian cancer patients; the 453 cells were from a breast cancer patient; 231 and SKBR3 are breast adenocarcinoma cell lines, and 5, SKOV3 and SKOVLB are adenomacarcinous ovarian cancer cell lines. The lines 1, 453 and SKBR3, resistant to ACP, were co-resistant to VT1. Fig. 5 shows VT sensitive and resistant cell lines tested for the presence of Gb_3 by VT binding in tlc overlay. Glycolipid from an equal number of cells were extracted and separated by tlc prior to toxin binding. In Fig. 5, lane 1:SKBR3, lane 2:468, lane 3:231, lane 4:453, lane 5 Gb_3 standard, lane 6:SKOV3, lane 7:SKOVLB. Cell lines SKBR3, 468, 231 and 453 are derived from breast tumours. Only 231 is sensitive to VT1. SKOVLB is a multiple drug resistant ovarian tumour cell line derived from SKOV3.

Ovarian tumour cells were highly sensitive to VT (Fig. 3) and contained elevated levels of the VT receptor, Gb_3 (Fig. 4). Breast cancer cells were for the most part, toxin resistant (Fig. 3) and receptor negative (Fig. 5). Low levels of Gb_3 were detected in normal ovarian tissue but these were markedly elevated for the ovarian tumour tissue samples.

The specific elevation of Gb_3 in ovarian tumours as opposed to normal ovary tissue provides the feasibility of using the toxin in the management of this malignancy. Ovarian tumours are often refractory to chemotherapy and prognosis is poor. Indeed, preliminary phase 1 clinical trials using a ACP injected directly into skin malignancies (Mycosis fungoides) have proven successful without adverse systemic effects.

With reference now to Fig. 6, human derived ovarian tumour cell lines were tested for VT1, VT2, and VT2c sensitivity. The cells were grown to confluence in 48-well

plates, then incubated for 48 hrs. in the presence of increasing doses of VTs. SKOVLB, the multiple drug resistant variant of SKOV3 ovarian line, showed the most sensitivity to VT's with SKOVLC being the next most sensitive to the VT's.

We have found that both drug resistant cells are approximately 500 to 1000 times more sensitive to verotoxin cytotoxicity than the parental SKOV3 cell line.

Fig. 7 shows the effect after 48 hrs. of treatment of the brain tumour SF-539 cell line derived from a recurrent, right temporoparictal glioblastoma multiform with VT1, VT2, and VT2c. This cell line, as others, was highly sensitive to VT's.

Fig. 8 provides the results from imaging a nude mouse with ^{131}I-VT1B (CPM distribution in different organs). VT1B-^{131}I cpm distribution in nude mouse with implanted ovarian tumour showed that a considerable amount of radiolabled VT1B had been concentrated in the ovarian tumour. Only a trace amount of VT1B was located in the brain where the potential VT1 side effect was considered. Since the lung in human adult is not the site of concern for VT1 toxicity this does not present a problem for treatment of human adult with ovarian tumour. In addition the CPM in kidney includes the excreted radiolabelled VT1 B subunit. Accordingly, based on this test, imaging with labelled VT1 B subunit can be a very useful method for screening the susceptible patient to VT1 cytotoxicity.

Fig. 9 shows the sensitivity of a variety of human astrocyta cell lines to VT1. All these cells contain Gb_3 but show variable sensitivity to VT1 induced cytotoxicity. This suggests that certain astrocytomas will be susceptible to verotoxin wheres others may not. This is important since astrocytomas are very refractory to treatment at the present time and cell sensitivity <u>in vitro</u> to concentrations as low as 5ng per/ml is rare.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

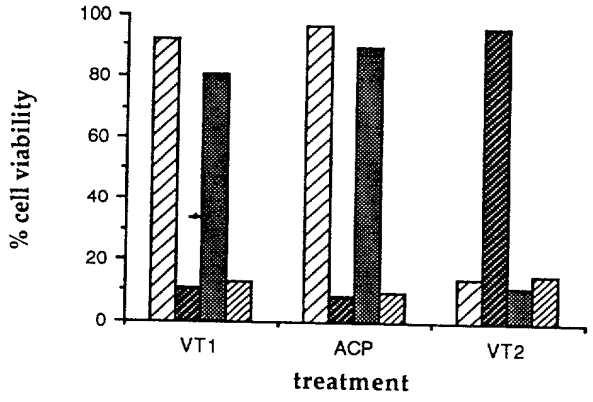
We claim:

1. A pharmaceutical composition for the treatment of mammalian Gb_3-containing neoplasia comprising a non-lethal, anti-neoplasia effective amount of a verotoxin and a suitable pharmaceutically acceptable diluent, carrier or adjuvant therefor.
2. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 1.
3. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 2.
4. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 2c.
5. A method of treating mammalian neoplasia comprising treating said mammal with a non-lethal, anti-neoplasia effective amount of a verotoxin.
6. A method as defined in Claim 3 wherein said verotoxin is verotoxin 1.
7. A method as defined in Claim 3 wherein said verotoxin is verotoxin 2.
8. A method as defined in Claim 3 wherein said verotoxin is verotoxin 2c.
9. A method as defined in Claim 5 wherein said neoplasia is ovarian cancer.
10. A method as defined in Claim 5 wherein said neoplasia is Mycosis fungoides.

Abstract of the Disclosure

Pharmaceutical compositions comprising known verotoxins, particularly, verotoxin 1, have been found to be useful in the treatment of mammalian neoplasia, particularly, ovarian cancer and skin cancer. Surprisingly, although verotoxin 1 has previously been shown to have anti-neoplastic activity *in vitro*, non-lethal doses of verotoxin 1 have been shown to be therapeutically anti-neoplastic *in vivo*.

fig 1



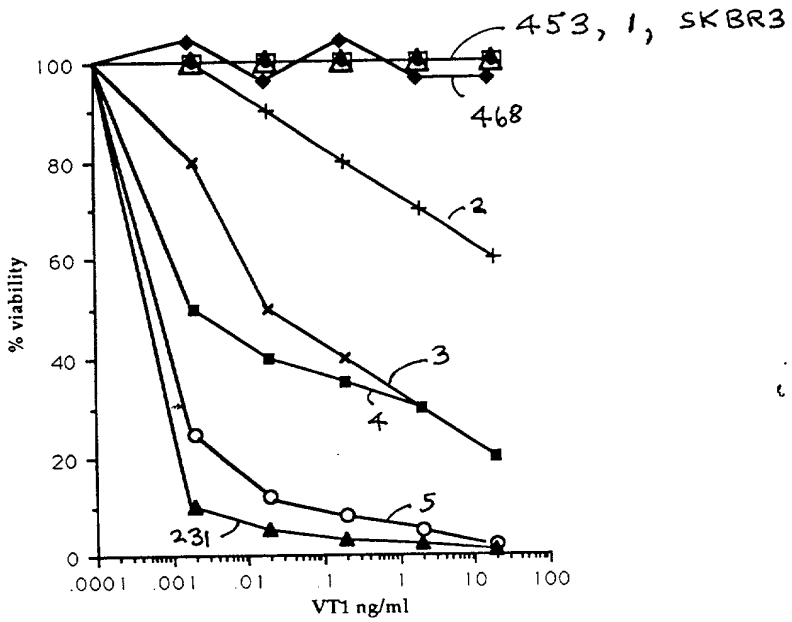


Fig. 2

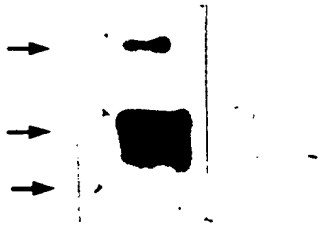


Fig. 3

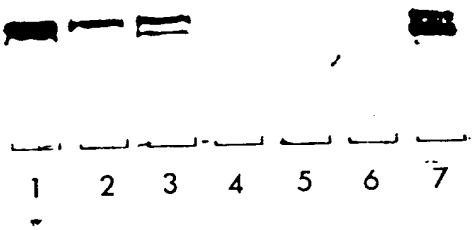


Fig. 4

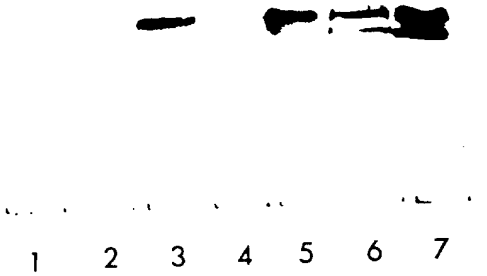
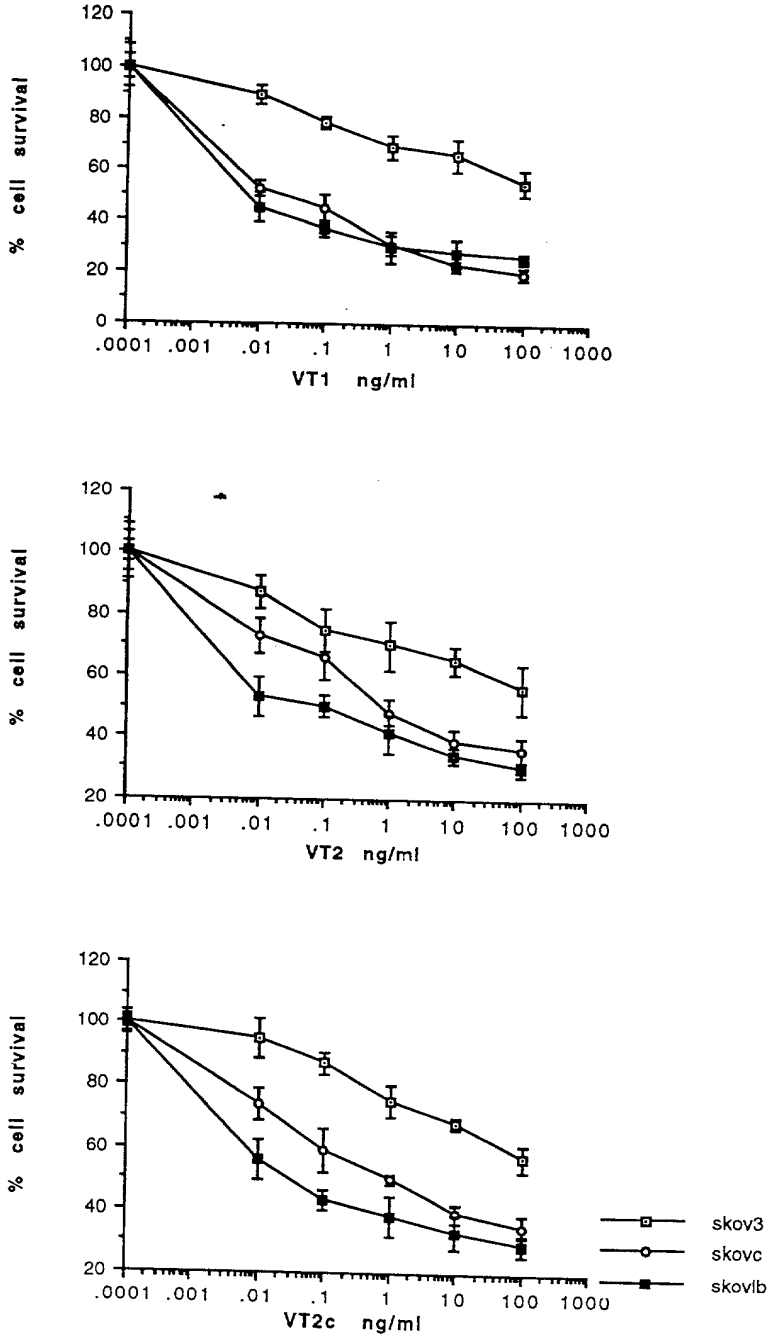


Fig. 5

Figure 6


% cell survival
120
100
80
60
40
20
0
.0001 .001 .01 .1 1 10 100 1000
VT1 ng/ml
% cell survival
120
100
80
60
40
20
.0001 .001 .01 .1 1 10 100 1000
VT2 ng/ml
% cell survival
120
100
80
60
40
20
.0001 .001 .01 .1 1 10 100 1000
VT2c ng/ml
skov3
skovc
skovlb

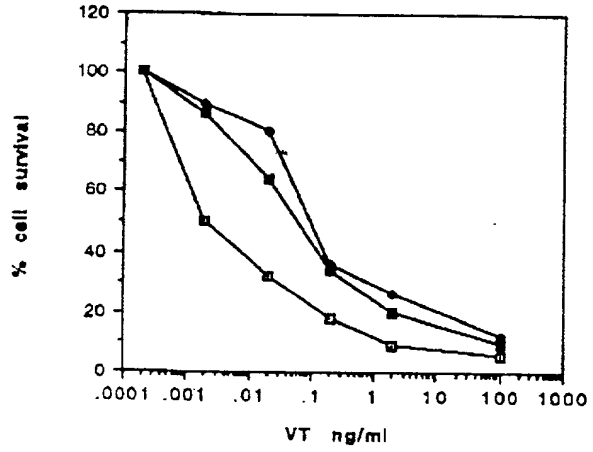


FIG 7

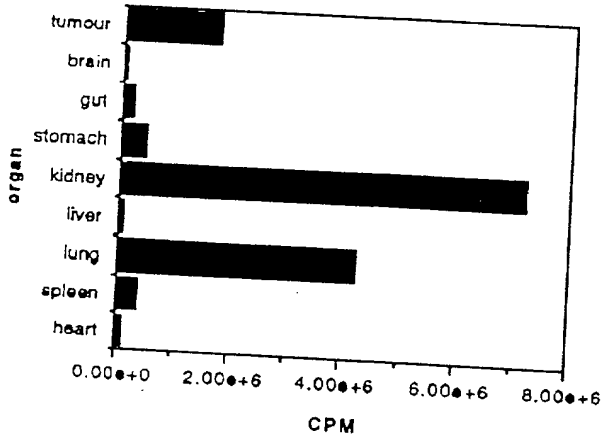


FIG 8

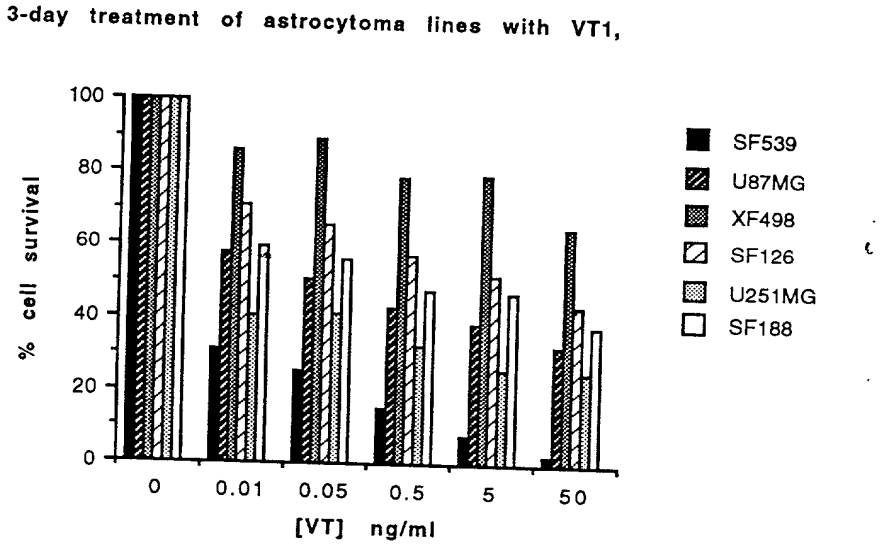

3-day treatment of astrocytoma lines with VT1,
% cell survival
100
80
60
40
20
0
0
0.01
0.05
0.5
5
50
[VT] ng/ml
SF539
U87MG
XF498
SF126
U251MG
SF188

Fig. 9

SL427

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VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND MEDICAL TREATMENTS THEREWITH

the specification of which (CHECK applicable BOX(ES))

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X -> ☐ was filed on _____ as U.S. Application No. 0_/_____

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I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C.119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

PRIOR FOREIGN APPLICATION(S) Number	Country	Day/MONTH/Year Filed	Date first Laid-open or Published	Date Patented or Granted	Priority Claimed Yes	No
2,116,179	Canada	22/02/1994			X	

I hereby claim the benefit under 35 U.S.C. 120/365 of all United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

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And I hereby appoint Cushman, Darby & Cushman, 1100 New York Avenue, N.W., Ninth Floor, Washington, D.C. 20005-3918 telephone number 861-3000 (to whom all communications are to be directed), and the below-named persons (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Cushman, Darby & Cushman in writing to the contrary.

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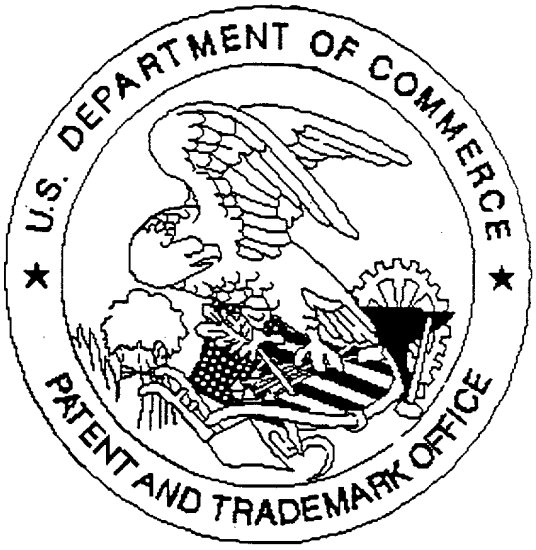

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